Bacterial fingerprints across Europe

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CHAPTER 6

High anti-staphylococcal antibody titers in patients with epidermolysis bullosa relate to long-term colonization with alternating types of *Staphylococcus aureus*

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ABSTRACT

Patients with the blistering disease epidermolysis bullosa (EB) develop wounds that are highly susceptible to bacterial colonization. Recently, we reported that over 75% of the EB patients sampled at one particular point of time were colonized with *Staphylococcus aureus*. To determine possible changes in *S. aureus* colonization over time, swabs were collected from the nares, throats and wounds of 61 EB patients at three time points during a period of ~2 years. All *S. aureus* isolates were typed by multiple-locus variable number tandem repeat analysis and *spa*-typing. This revealed major fluctuations in the *S. aureus* types sampled from individual EB patients. In addition, blood donations were obtained from 13 EB patients to determine their immunoglobulin (IgG) levels against 43 virulence factors or whole cells of *S. aureus*. Overall, the sera of EB patients contained higher anti-staphylococcal IgG levels than those of healthy individuals. Specifically, this applied to IgGs against nine important virulence factors, including the superantigens SEM, SEN and SEO. Notably, EB patients carrying different *S. aureus* types contained higher levels of anti-staphylococcal antibodies than EB patients colonized by only one type. Our findings suggest that the immune system of EB patients is heavily challenged with *S. aureus* antigens.

Epidermolysis bullosa (EB) is a genetic blistering disease that renders patients susceptible to colonization by the opportunistic pathogen *Staphylococcus aureus* [1-3]. Recently, we observed that all EB patients with chronic wounds, and 75% of the patients without chronic wounds were colonized with *S. aureus* [4]. In contrast, only ~30% of the healthy human population carries this pathogen [5]. Persistent *S. aureus* carriers have an increased risk for staphylococcal infections but, compared to non-carriers, their risk of death due to bacteraemia is lower [6]. This may relate to increased levels of protective anti-staphylococcal antibodies upon long-term exposure to colonizing strains [7]. Furthermore, anti-staphylococcal antibody levels were shown to increase strongly during bacteraemia [7,8]. Since high exposure to *S. aureus* is a potential health risk for EB patients, our present studies were firstly aimed at defining their *S. aureus* population over time and, secondly, at determining their anti-staphylococcal immunoglobulin (IgG) levels.

Based on informed consent, 61 EB patients from the Dutch Epidermolysis Bullosa Registry were included in our studies (Supplementary Materials and Methods under doi:10.1038/jid.2012.347 or available upon request). *S. aureus* colonization was determined in three rounds of sampling at half-yearly intervals. In each round, swabs were collected from 3 wounds, the left and right anterior nares, and the throat. 43 EB patients participated in the second sampling round, 40 in the third, and 35 patients participated in all three sampling rounds. Overall, we identified 101 different *S. aureus* types by molecular typing with multiple-locus variable number tandem repeat analysis (MLVA; Supplementary Material and Methods and Table S1, under doi:10.1038/jid.2012.347 or available upon request). Only 18 of these MLVA types were encountered in all rounds (Figure 1A). 118 strains were also *spa*-typed, revealing 48 different *spa*-types (Table S1). Next, we compared the variations in *S. aureus* types isolated from individual EB patients over time. This revealed that the same MLVA type was identified on ~42.5% of all sampled patients with minor variations for different sites of sampling (Figure 1B). Furthermore, 58.3% of the patients with chronic wounds and 43.5% of the patients without chronic wounds carried alternating *S. aureus* MLVA types over time. In 8.7% of the patients without chronic wounds, a different MLVA type was encountered in each sampling round. These findings show that the included EB patients are continuously challenged by different *S. aureus* types and that the carried *S. aureus* population can change rapidly. This seems to challenge the classical dogma that persistent carriers are mainly colonized by one *S. aureus* type [5]. However, our studies specifically address a patient group that is highly susceptible to *S. aureus* due to continuous skin defects, which is different from the situation in healthy individuals.

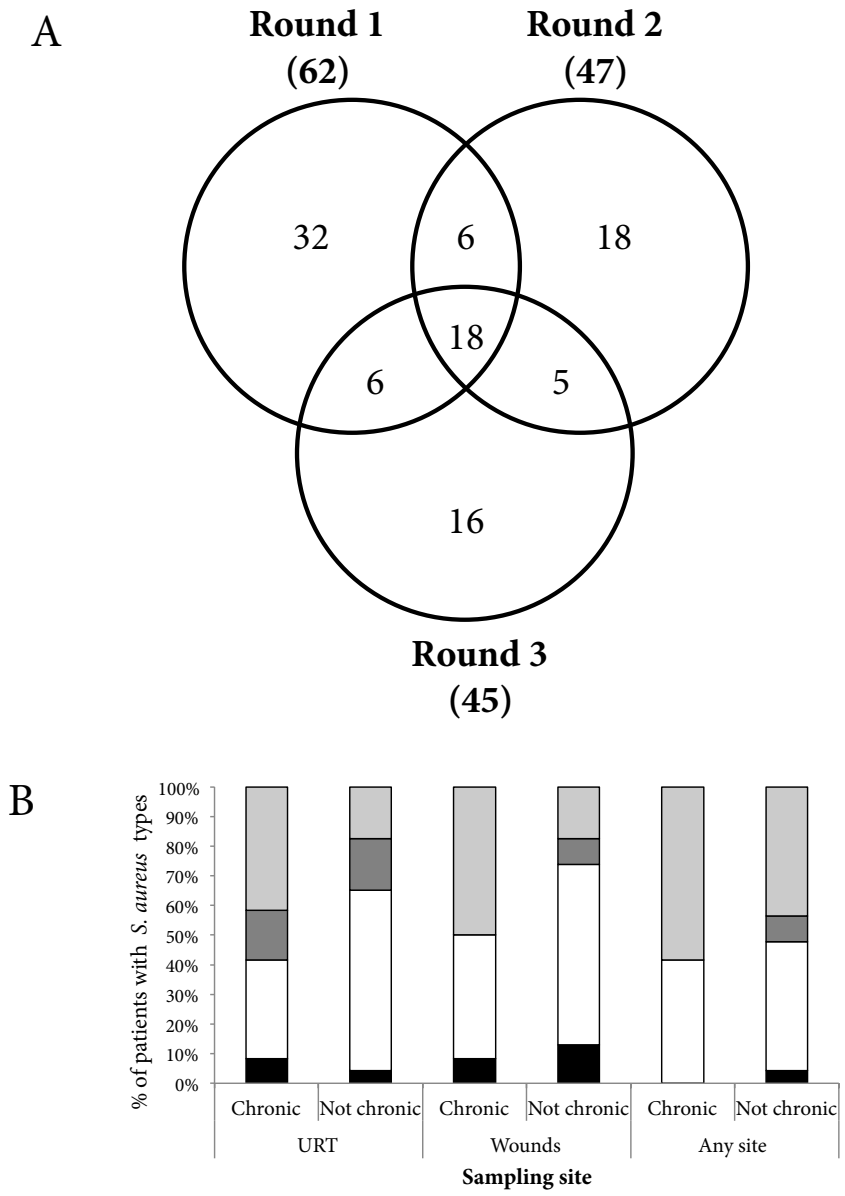

Figure 1. *S. aureus* MLVA types identified in EB patients over a period of ~2 years. (**A**) Summary of the numbers of different MLVA types identified in three rounds of sampling. (**B**) Changes in the *S. aureus* MLVA types isolated from 35 EB patients in three rounds of sampling. The MLVA typing results were analysed for individual EB patients with chronic wounds (n = 12) or without chronic wounds (n = 23). Black bars, percentage of patients not carrying *S. aureus*; white bars, percentage of patients colonized by the same MLVA type in all three sampling rounds; dark grey bars, percentage of patients colonized by different types in all three sampling rounds; light grey bars, percentage of patients with alternating MLVA types. URT, upper respiratory tract.

To assess the anti-staphylococcal IgG levels in EB patients, we first performed whole-cell enzyme-linked immunosorbent assays (ELISAs) using an *S. aureus* mutant lacking the IgG-binding proteins Sbi and protein A, and IgGs isolated from patients with chronic wounds. Sera from healthy donors were used as controls. This revealed that EB patient sera contained significantly higher anti-staphylococcal IgG levels than the controls (Supplementary Figure S1, under doi:10.1038/jid.2012.347 or available upon request). To determine specific responses, the levels of serum IgGs from 13 EB patients against 43 purified *S. aureus* antigens were measured using Luminex technology (Figure 2). As controls, the sera from 14 age-matched healthy individuals were used. For most antigens, the median fluorescence intensities (MFI) were higher in EB patients than in the control group. Especially, the MFI levels for IgGs against the surface proteins IsdA and SasG, the secreted proteins IsaA, SCIN, Nuc and LytM, and the staphylococcal superantigens (SAgs) SEM, SEN and SEO were statistically significantly higher in EB patients. The increased IgG levels against IsaA, SCIN, Nuc, and LytM could be explained by the fact that these proteins are expressed by many *S. aureus* types [9]. Also, the *egc* gene cluster-encoded SAgs SEM, SEN and SEO are amongst the most prevalent SAgs of *S. aureus* (52%-66%) [10]. Intriguingly, persistent carriers, bacteraemia patients and furunculosis patients were found to develop no, or only low levels of antibodies against these SAgs [11-13]. This suggests that EB patients are more significantly challenged by *egc* SAgs than healthy carriers and bacteraemia or furunculosis patients.

To determine whether carriage of multiple *S. aureus* strains impacts on anti-staphylococcal IgG levels, we compared patients colonized by one MLVA type (n= 7) with patients colonized by multiple MLVA types (n=5). Interestingly, the highest MFI levels were observed for IgGs from patients carrying multiple MLVA types. This was particularly evident for IgGs against IsdA, LukD, HlgB, LytM, LukS, LukF and ETA (Figure 2B). Notably, the incidence of LukS/F is very low so, conceivably, the respective Luminex signals represent cross-reactive IgGs against the more common HlgA/B or LukE/D proteins [14]. A significant correlation between anti-staphylococcal IgG levels in serum, wound fluid and sterile blister fluid was revealed in samples from one EB patient (Supplementary Figure S2, under doi:10.1038/jid.2012.347 or available upon request). Here, the largest difference concerned 4-fold lowered anti-IsaA levels in wound fluid. This implies that future studies on anti-staphylococcal immune responses in EB patients can be based on non-invasively sampled wound fluid.

In conclusion, EB patients are highly challenged with very diverse *S. aureus* types and carriage of multiple *S. aureus* types seems to elicit the highest humoral responses in these patients. However, we cannot exclude the alternative possibility of increased humoral- and reduced cell-mediated immunity in EB patients, which might impact on *S. aureus* carriage. Notably, EB patients do not frequently suffer from *S. aureus* bacteraemia, and none of the patients who donated blood was treated for staphylococcal bacteraemia in the five years prior blood donation. This suggests that their high anti-staphylococcal antibody titres may be protective against invasive *S. aureus* infections, which would be consistent with the protective effects of IsaA-specific antibodies in mice [15].

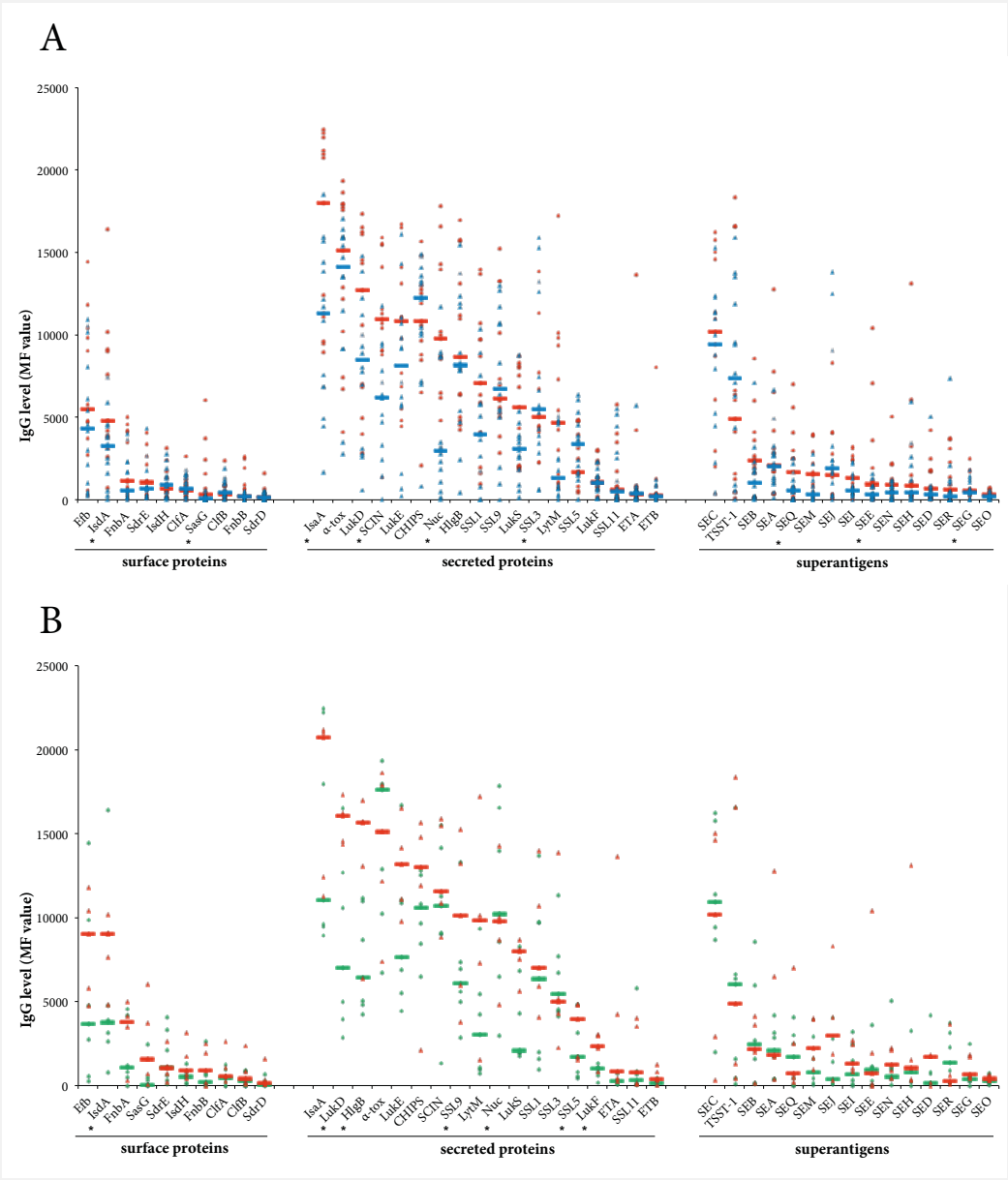

Figure 2. IgG responses of EB patients to staphylococcal antigens. (**A**) IgG levels against 43 purified *S. aureus* antigens in sera of EB patients (red diamonds; n = 13) or age-matched healthy controls (blue triangles; n = 14) were determined by Luminex assays. Median fluorescence intensity (MFI) values as indicated by color-coded bars (EB patients, red; healthy controls, blue) reflect the levels of antigen-specific IgGs. (**B**) IgG levels against 43 *S. aureus* antigens in sera of EB patients colonized by multiple MLVA types (red triangles; n = 5), or EB patients colonized by only one MLVA type (green diamonds; n = 7) were determined by Luminex assays. The respective MFI values are indicated by red and green bars.

ABBREVIATIONS

Clf, clumping factor; EfB, extracellular fibrinogen-binding protein; ET, exfoliative toxin; Fnb, fibronectin binding protein; HlgB, gamma-hemolysin B; IsaA, immunodominant antigen A; Isd, iron-responsive surface determinant; Luk, leukocidin; LytM, peptidoglycan hydrolase; Nuc, endonuclease; SasG, *S. aureus* surface protein G; SCIN, staphylococcal complement inhibitor; CHIPS, chemotaxis inhibitory protein of *S. aureus*; Sdr, serine-aspartate dipeptide repeat protein; SE, staphylococcal enterotoxin; SSL, staphylococcal superantigen-like protein; TSST-1, toxic shock syndrome toxin.

CONFLICT OF INTEREST

The authors state no conflict of interest. However, it should be noted that GSE, HW, HPJB and HG are employees of IQ Therapeutics.

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‘Fange nie an aufzuhören, höre nie auf anzufangen.’